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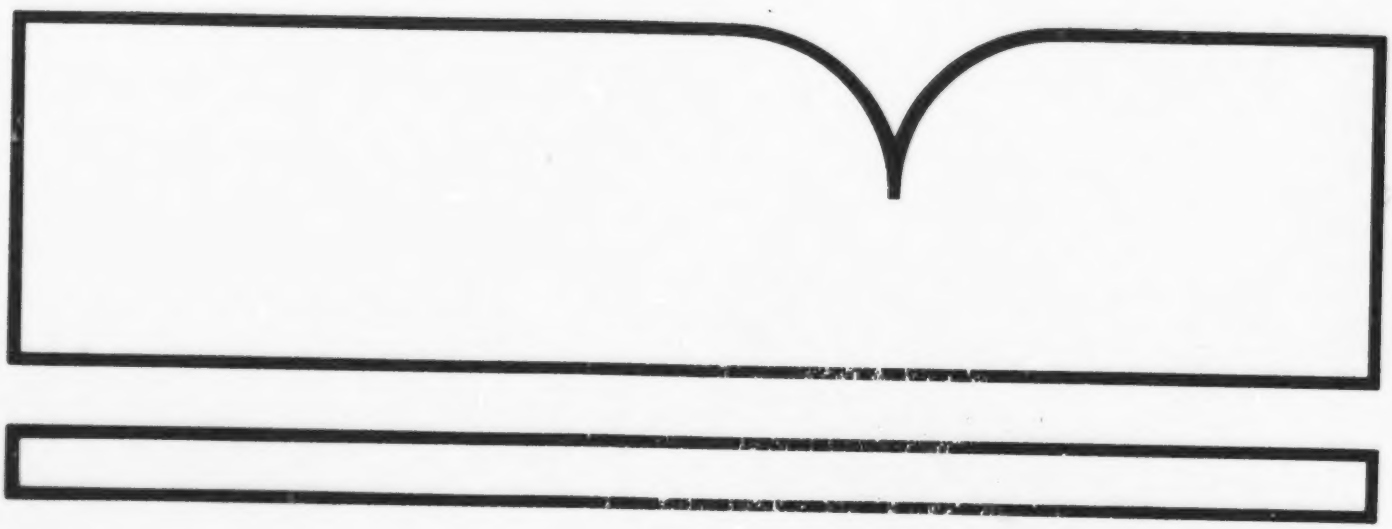

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FACTORS DETERMINING THE IN VITRO RESPONSE OF RODENT EMBRYOS TO TERATOGENS

by

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16. ABSTRACT

Four teratogens, with different modes of action and pharmacological properties, were selected and investigated in the embryo culture system. The compounds included hydroxyurea (HU), cadmium (Cd), diphenylhydantoin (DPH), and cyclophosphamide (CPP). The overall goal of the study was to characterize the response of early stage (2-6 somites) mouse and rat embryos to these agents in an effort to test the potential of utilizing the system of whole embryo culture for basic teratological and toxicological investigations and as a screening technique for teratogens. The paper summarizes the results of studies with these 4 compounds.

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INTRODUCTION

For the past several years our laboratory has been concerned with the further development and characterization of the rotating system of whole embryo culture as originally designed by Denis New ('78; Sadler, '79) (Sadler et al 1982; Warner et al, 1983; 1984; Sadler and Warner, 1984). Further development of the system has been directed toward simplification and the use of commercially available inexpensive apparati such that any laboratory can readily establish and employ the technique (Sadler, '79, Sadler et al, '82). Characterization of the system has focused on a comparison of the in vitro versus in vivo response to teratogens and on the role of pharmacokinetics, drug metabolism, stage of development, etc. in determining the effects of teratogenic agents on morphogenesis (Warner et al, '83, '84; Sadler and Warner, '84). Toward this end 4 teratogens, with different modes of action and pharmacological properties, were selected and investigated in the system. The compounds included hydroxyurea (HU), cadmium (Cd), diphenylhydantoin (DPH), and cyclophosphamide (CPP). The overall goal of the study was to characterize the response of early stage (2-6 somites) mouse and rat embryos to these agents in an effort to test the potential of utilizing the system of whole embryo culture for basic teratological and toxicological investigations and as a screening technique for teratogens.

METHODS

Investigations with each of the compounds were conducted similarly. Thus, the first step was to establish a teratogenic dose in vivo in rats and/or mice by administering a single intraperitoneal injection of the agent during the early somite stage of gestation (9th day in the ICR

mouse; 10th day in the Sprague Dawley rat; plug day = day 1). Dose response curves for each compound were established and the dose chosen for all subsequent studies was the one that provided approximately a 50% malformation rate or was the maximally tolerated dose by the mother.

Using this dose, maternal serum concentrations of the administered compound were monitored at various times after treatment to determine the peak serum concentration and the serum half-life (t1/2) of the agent. Assays of HU were conducted using a modification of the Nery technique (Nery, '66); Cd by flame absorption spectrometry; DPH by a fluorometric procedure (Ames, TDA Series, Miles Laboratories, Inc., Elkhart, Indiana); and CPP by a modification of the 4-(4'-nitrobenzyl)-pyridine method (Christian et al, '80).

In vitro studies involved the use of whole embryo culture as described previously (Sadler, '79) and employed early somite stage (2-6) mouse and rat embryos (9 and 10-day embryos, respectively, to correspond to the in vivo days of treatment). Two to three embryos were placed into culture vials containing control medium (2.25ml. of rat serum and 0.75ml. Tyrode's solution) and the proper gas mixture (5% 02, 5% CO2, 90%N2) for 30 min. Following this equilibration period, the compound to be tested was administered through the cap (rubber stopper) of the culture vial using a microliter syringe and the flask returned to the incubator (37^{o}) for the appropriate exposure time. After exposure, embryos were removed from the flasks, washed 3x in sterile Tyrode's solution, and placed individually into flasks containing control medium. All cultures were then maintained for a total time of 48h, (Figs. 1 and 2).

For each compound, the concentration and exposure time used for in vitro studies were based on results from the in vivo pharmacokinetics investigations and were defined as the peak serum value obtained following

administration of the agent in vivo at the teratogenic dose, for the t1/2 of the compound. For example, the peak serum value obtained in mice in vivo for a teratogenic dose (300 mg/kg) of HU was 300 ug/ml. and the serum t1/2 was 30 min. Therefore, embryos grown in vitro were exposed to this concentration for 30 min. followed by transfer to control medium for the remainder of the total 48h culture period. Permutations of this original protocol were then employed to test the effects of concentration versus time of exposure on the embryo's response to teratogens in vitro. Thus, studies were conducted in which 0.5 to 3 times the peak serum concentrations and/or 2 times the t1/2 of a compound were employed.

In addition to these basic investigations, 3 other approaches, designed to further characterize the rodent embryos' response to teratogens in whole embryo culture, were employed: 1). Effects of a subteratogenic dose of a compound, in this case Cd, were assessed to test the sensitivity of the in vitro versus in vivo embryonic response. A subteratogenic dose of Cd (1.0 mg/kg) was administered in vivo, which produced less that a 10% rate of malformations. Peak serum values and the serum half-life of Cd following this dose were determined and these conditions were reproduced in culture (1.25 ug/ml. for 30 min.); 2). An example of the interaction of compounds was tested by determining the capacity for zinc (Zn) amelioration of Cd-induced defects in vitro. This effect has been well-documented in vivo (Ferm and Carpenter, '68; Garcia and Lee, '81) and was tested in whole embryo culture by pre-incubating embryos in Zn at approximately 3 times the molar concentration of a teratogenic dose of Cd (150uM Zn, 50 uM Cd). Cadmium was then added to the same medium and the embryos exposed for 30 and 60 min., i.e., the serum t1/2 and twice the t1/2 following a teratogenic dose of Cd in vivo;

3). Finally, in an attempt to assess the effects of metabolic activation of a compound, the peak serum levels of CPP as well as the alkylating activity contained in that serum following the in vivo administration of a teratogenic dose of the agent (10 mg/kg in rats) were determined. Cyclophosphamide was then added directly to cultures and tested for teratogenicity. In addition rats were dosed with the compound and then their serum was collected, monitored for alkylating activity, and then used as culture medium.

RESULTS

The compounds used in the study were selected for specific reasons: HU and Cd because they are known to have a direct effect on the conceptus and their teratogenicity was well documented (Scott et al, '71' Ferm, '71); DPH because, although it is teratogenic, it affects later stages of development (cleft lip and palate) and shows strain differences (Harbison and Becker, '69); CPP because it requires "metabolic activation" to exert its teratogenic effects, as has been well documented by several investigators (Fantel et al., '79; Klein et al., '80). By selecting such a diverse group of compounds, the sensitivity, as well as stage and species specificity, of the in vitro embryonic respone to teratogens could be compared to their response in vivo.

Results from the initial series of experiments employing the peak serum concentration and t1/2 protocol are provided in Table 1. As illustrated, in some cases this protocol reproduces the in vivo response, for example, with HU and DPH. On the other hand, Cd both with respect to percent malformations and effects on growth (total protein) produces a greater teratogenic response in vitro than in vivo using this protocol. Despite this greater sensitivity in vitro, however, the sensitivity

between species was maintained with rat embryos responding at much lower doses than mice both in vivo and in vitro. Cyclophosphamide, as reported previously, (Fantel et al, '79; Klein et al., '80) has no direct effect on the embryo, even at concentrations approximately 15 times higher than the peak serum concentration obtained following a teratogenic dose in vivo.

In addition to monitoring the malformation rates and total protein values following exposure to the teratogens, the types of malformations produced were also recorded. In all cases the in vivo versus in vitro response was similar. For example, HU produced predominently cranial neural tube defects (exencephaly) in vivo and in culture (Warner et al., '83), whereas Cd induced craniofacial abnormalities, including micropthalmia and midline craniofacial clefting (Figs. 3 and 4).

Using the compounds HU and Cd, the effects of dose and exposure time (concentration = peak concentration value following an in vivo teratogenic dose; time = the serum t1/2) on the teratogenic response in vitro were also investigated. The results are summarized in Table 2 and clearly demonstrate that both parameters are significant in influencing malformation rates and embryonic growth. For example, HU at either 2 times the concentration (600 ug/ml) or 2 times the half-life (1h exposure time) produced a similar response in the embryos, with a 100% malformation rate and approximately a 50% reduction in total protein (Warner et al., '83). Results with Cd were similar and in fact doubling the exposure time (1h) at the peak serum concentration (5.35 ug/ml) killed all the embryos (Warner et al., '84).

The question of embryonic sensitivity in culture was addressed by determining a subteratogenic dose of Cd in vivo that produced less than a 10% rate of malformations. Peak serum concentrations attained after this

dose and the serum t1/2 were determined and these conditions reproduced in culture (1.26 ug/ml, 0.5h exposure time). The in vitro effects were similar to those obtained in vivo and embryos were not malformed (Table 3). Varying this dose and exposure time in culture produced results similar to those depicted in Table 2 and in fact demonstrated that exposure time was more critical than the concentrations employed.

The potential for employing the culture system to test the interaction between compounds was assessed using Zinc (Zn) to protect the embryos from the teratogenic affects of Cd. This protection has been well documented in vivo (Ferm and Carpenter, '68; Garcia and Lee, '81) and also operates in vitro (Warner, et al., '84). For example, placing Zn in the culture medium prior to the addition of Cd significantly reduced the rate of Cd-induced malformations and growth inhibition. Furthermore, even with a treatment protocol whereby Cd killed 100% of the embryos, the addition of Zn to the medium permitted embryonic survival and several embryos developed normally (Table 4).

Finally, the requirement for bioactivation of compounds was demonstrated by again employing CPP and basically repeating studies conducted by Fantel et al.('79) and Klein et al.('80). The results achieved were similar to those obtained by these 2 laboratories as it was shown that the parent compound has no teratogenic activity, even when added at 15 times the peak serum concentration observed following a teratogenic dose in vivo. However, serum obtained from rats injected with CPP did have teratogenic activity (presumably because it contains the active metabolite(s), although exposure times required to produce a significant malformation rate were quite long, 5-25h, compared to the t1/2, 2.5h, of the alkylating activity of the compound in vivo. In this regard, the teratogenic parameters monitored in this series of experiments

was alkylating activity in the serum. Alkylating metabolites such as phosphoramide mustard are generated from the parent compound and have teratogenic actions . However, 2 observations regarding alkylating activity are noteworthy in these experiments: 1). Based on the fact that exposure times in vitro were extended well beyond the serum half-life of the compound, alkylating activity may not be the only parameter responsible for producing malformations; 2). Heat inactivation of serum reduced the alkylating activity by approximately one-third, necessitating the administration of a higher in vivo dose of the compound to achieve the appropriate levels of this activity in serum used for culture.

DISCUSSION

This series of studies clearly demonstrates the system of whole embryo culture's potential for use in teratological and toxicological studies and perhaps as a screening technique as well. Under the appropriate conditions, rodent embryos respond in a manner similar to their response in vivo. Thus, parameters such as concentration, time of exposure, stage of development, etc. must be considered in designing experiments. Furthermore, the system is not overly sensitive and a subteratogenic dose in vivo produces a similar response in vitro. Also, DPH, a compound that is not teratogenic at this stage of development in vivo, is not teratogenic in culture, when conditions approximating those observed in vivo are established. Ultimately, all of these factors including dose, exposure time, and stage of development must be considered in the design of studies involving mechanisms of teratogenesis or even for the screening of potential teratogens.

The system is also capable of detecting the metabolic activation of a

compound, such as CPP. Whether this activation is achieved by adding a drug metabolizing system to the cultures (Fantel et al., '79) or by administering the agent to an adult animal and then using serum collected from this specimen as culture medium (Klein et al., '80), similar results have been obtained. Both techniques are useful, although the present study has shown that procedures necessary for the preparation of the serum as culture medium, i. e., heat inactivation, can significantly alter the concentration of metabolites and should be considered.

As far as its usefulness as a screening technique for teratogens, the embryo culture system has many attributes. It employs mammalian embryos which respond to teratogens in a manner similar to their in vivo counterparts, i.e., show responses to dose and time of exposure. The time required for obtaining results is rapid, and the cost in animals and personnel is less than that encountered in in vivo testing procedures (Sadler et al., '82; Sadler and Warner, '84). In addition, the system has the advantage of being able to screen serum from primates and humans for toxic and teratogenic factors (by employing these subjects' serum as culture medium and determining its effects on rodent embryogenesis). This latter approach has been employed extensively by Klein's laboratory and has met with substantial success (Chatot et al., '80; Klein et al., '82).

In summary, the potential of whole embryo culture to serve as a screening technique for teratogens is rapidly being documented and only awaits validation. Furthermore, the systems' significance for the science of teratology and developmental biology is unquestionable. Mechanisms studies in the former and a host of studies, such as tissue interactions and transplants in the latter, are possible.

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Table 1: IN VIVO AND IN VITRO EFFECTS OF TERATOGENS

Teratogen	Dose[1] mg/kg ug/ml	T 1/2[2]	%Malformations in vivo	%Malformations in vitro	Protein (ug)[3]
Control(mice)	-	-	1		348±54
Control(mice)	-	-		3	346±63
Control(rat)	-	-	2		340±81
Control(rat)	-	-		5	310±66
HU	300	30m	45		153±132[4]
HU	300	30m		30	174±85[4]
Cd (mice)	6.0	30m	57.5		287±118
Cd (mice)	5.35	30m		85.7	161±104[4]
Cd (rat)	4.0	40m	59		180±93[4]
Cd (rat)	3.2	40m		100	74±26[4]
DPH	85	32h	10		395±117
DPH	32.5	32h		10	294±62
CPP (mice)	15	30m	80		171±129[4]
CPP (mice)	3768	30m		9	344±88
CPP (rat)	10	80m	100		102±50[4]
CPP (rat)	3989	80m		0	340±64

[1] Dose represents an in vivo teratogenic or maximally tolerated administration of the compound (mg/Kg) versus the concentration employed in vitro (µg/mg) which represents the peak serum concentration attained following a teratogenic dose in vivo.

[2] T 1/2 represents the serum half life of each compound at the teratogenic or maximally tolerated dose in vivo.

[3] Mean ± SD

[4] P <.001 compared to controls, Students t-test.

Table 2: EFFECTS OF DOSE AND TIME OF EXPOSURE (Mice)

Teratogen	Dose	T 1/2	%Malformations	Protein[1]
Control	-	-	3	346±63
HU	X[2]	X[3]	30	237±77[5]
HU	2X	X	100	151±60[4]
HU	X	2X	100	125±16[4]
HU	1/2 X	X	6	249±56
Cd	X	X	86	161±105[4]
Cd	1/2 X	X	72*	230±90[4]
Cd	X	2X	100	FTT

[1] Mean ± SD

[2] X Dose = peak serum concentration of HU (300 µg/ml) and Cd (5.35 µg/ml) attained following a teratogenic dose of each compound (HU = 300 mg/Kg; Cd 6 mg/Kg) in vivo.

[3] X time = serum t 1/2 of each compound (30 min.) following the same teratogenic dose in vivo.

[4] P <.001, Students t-test.

[5] P <.05

Table 3: IN VIVO VERSUS IN VITRO EFFECTS AT SUBTERATOGENIC DOSES IN MICE

Teratogen	Dose	T 1/2	%Malformations in vivo	%Malformations in vitro	Protein (ug) [1]
Control	-	-		3	346±63
Cd	1 mg/kg	30m	2(42)		-
Cd	1.25 ug/ml [2]	30m [3]		12(16)	301±58
Cd	3.75 ug/ml	30m		17(6)	212±42 [4]
Cd	1.25 ug/ml	60m		33(8)	183±29 [4]

[1] Mean ± SD

[2] Represents the peak serum concentration of Cd attained following a 1 mg/Kg dose in vivo.

[3] Represents the serum t 1/2 of Cd following a 1 mg/Kg dose in vivo.

[4] $P < .001$ compared to controls, Students t-test.

Table 4: ZINC PROTECTION OF CADMIUM TERATOGENESIS IN VITRO IN MICE

Treatment	%Malformations	Protein (ug)[1]
Control	0	355±34
Cd (xtime,xdose)[2]	89.5(N=19)	206±105[3]
Cd + Zn (10ug/ml)	14.2(N=14)	282±106
Cd (2xtime,xdose)	100(N=9)	FTT
Cd + Zn (10ug/ml)	64.3(N=14)	179±118[4]
Zn (10 ug/ml)	0(N=7)	364±44

[1]Mean ± SD

[2]xtime = the peak serum concentration of Cd (5.35ug/ml) attained following a teratogenic dose (6mg/Kg) in vivo: x dose = the serum t 1/2 of Cd (30 min.) following such an in vivo dose.

[3]P<.05 compared to controls, Students t-test

[4]P<.001 compared to controls

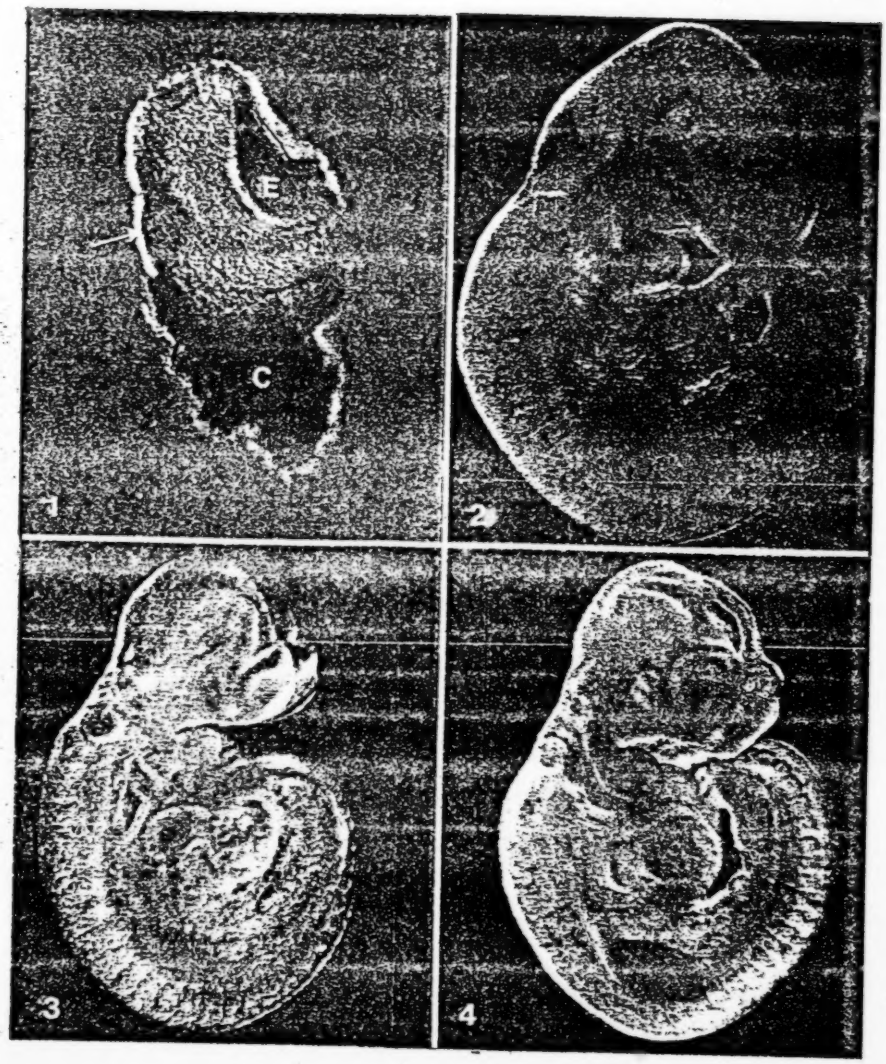
FIGURE LEGENDS

Fig. 1. Early somite (4 somites) mouse embryo as prepared for culture with the visceral yolk sac (arrow), embryo (E) and ectoplacental cone (C).

Fig. 2. Embryo (30 somites) after 48h of culture with the visceral yolk sac removed. The degree of morphogenesis is equivalent to that achieved in vivo during the same period.

Fig. 3. Eleventh day mouse embryo showing the characteristic craniofacial abnormalities induced by exposure to a teratogenic dose of $CdCl_2$ (6 mg./Kg) on the 9th day of gestation, (compare to Figs. 2 and 4).

Fig. 4. Embryo cultured 48h (9th-11th day) in the presence of $CdCl_2$ and showing similar craniofacial abnormalities as the embryo (Fig. 3) exposed in vivo. The in vitro concentration of $CdCl_2$ (5.35 ug/ml.) represented the peak serum level attained following a teratogenic (6 mg/Kg) dose in vivo. The exposure time (30 min.) represented the $t_{1/2}$ of Cd in the serum after an in vivo teratogenic dose.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